

Hydrogen Peroxide Colorimetric/Fluorometric Assay Kit

(Catalog #K265-200; 200 reactions; Store kit at -20°C)

I. Introduction:

Hydrogen Peroxide is a reactive oxygen metabolic byproduct that serves as a key regulator for a number of oxidative stress-related states. Functioning through NF-κB and other factors, hydroperoxide-mediated pathways have been linked to asthma, inflammatory arthritis, atherosclerosis, diabetic vasculopathy, osteoporosis, neuro-degenerative diseases, Down's syndrome and immune system diseases. BioVision's Hydrogen Peroxide Assay Kit is a highly sensitive, simple, direct and HTS-ready colorimetric and fluorometric assay for measuring H₂O₂ in biological samples. In the presence of Horse Radish Peroxidase (HRP), the OxiRed Probe reacts with H₂O₂ to produce product with color (λ_{max} = 570 nm) and red-fluorescent (Ex/Em = 535/587 nm). The kit can perform 200 reactions by fluorometric method or 100 reactions by colorimetric method. The detection limit can be as low as 2 pmol/well (or 40 nM) of H₂O₂ in the sensitive fluorometric assay.

II. Kit Contents:

Components	K265-200	Cap Code	Part Number
H ₂ O ₂ Assay Buffer	25 ml	WM	K265-200-1
OxiRed™ Probe (in anhydrous DMSO)	0.2 ml	Red	K265-200-2A
HRP	1 vial	Green	K265-200-4
H ₂ O ₂ Standard (0.88 M)	0.1 ml	Yellow	K265-200-5

III. Storage and Handling:

Warm the assay buffer to room temperature before use. Briefly centrifuge vials prior to opening. Read the entire protocol before performing the assay.

IV. Reagent Reconstitution and General Consideration:

OxiRed™ Probe: Ready to use as supplied. Briefly warm at 37°C to melt frozen DMSO. The OxiRed™ Probe solution is stable for 1 week at 4°C and 1 month at -20°C.

HRP: Dissolve in 220 µl Assay Buffer, pipetting up and down. The HRP solution is stable for 1 week at 4°C and 1 month at -20°C.

V. Hydrogen Peroxide Assay:

1. Sample Preparations:

Collect cell culture supernatant, serum, plasma, urine and other biological fluids (contains 0.8 - 6 µM H₂O₂). Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Remove particulate pellet. Samples, especially those such as culture medium, tissue lysate or plasma should be filtered through a 10 kDa MW spin filter (BioVision, Cat 1997-25) to remove all proteins then kept at -80°C for storage. It is recommended with all sample types to assay immediately or aliquot and store the samples at -80°C. Avoid repeated freeze-thaw cycles. Add 2 - 50 µl samples into each well, bring the volume to 50 µl with assay buffer.

2. H₂O₂ Standard Curve:

For the Colorimetric Assay: Dilute 10 µl 0.88 M H₂O₂ standard into 870 µl dH₂O to generate a 10 mM H₂O₂ standard, then dilute 10 µl of the 10 mM H₂O₂ standard into 990 µl dH₂O to generate a 0.1 mM H₂O₂ standard. Add 0, 10, 20, 30, 40, 50 µl of the 0.1 mM H₂O₂ standard into 96-well plate in duplicate to generate 0, 1, 2, 3, 4, 5 nmol/well H₂O₂ standard.

For the Fluorometric Assay: Dilute 100 µl of the 0.1 mM H₂O₂ standard into 900 µl dH₂O to generate a 10 µM H₂O₂ Standard. Add 0, 10, 20, 30, 40, 50 µl of the 10 µM H₂O₂ standard into a 96-well plate in duplicate to generate 0, 0.1, 0.2, 0.3, 0.4, 0.5 nmol/well H₂O₂ standard.

3. Reaction Mix: Mix enough reagents for the number of assays to be performed. For each well, prepare a total 50 µl Reaction Mix:

Colorimetric Assay

46 µl Assay Buffer
2 µl OxiRed™ Probe solution
2 µl HRP solution

Fluorometric Assay

48 µl Assay Buffer
1 µl OxiRed™ Probe solution
1 µl HRP solution

Add 50 µl of the Reaction Mix to each test samples and H₂O₂ standards. Mix well. Incubate at room temperature for 10 min.

*For a more sensitive assay, you can dilute the standard 10 fold further, decrease OxiRed™ amount to 0.2 µl and HRP amount to 0.4 µl per well, it will decrease the fluorescence background and detects as low as 2 pmol/well (or 40 µM) H₂O₂.

4. Measure OD (570 nm) or fluorescence (Ex/Em = 535/587 nm) in a micro-plate reader.

5. Calculation: Correct background by subtracting the value derived from the 0 nmol H₂O₂ control from all sample and standard readings (Note: The background reading can be significant and must be subtracted from sample readings). Plot the H₂O₂ standard curve. Apply your sample readings to the standard curve. H₂O₂ concentrations of the test samples can then be calculated,

$$C = Sa/Sv \text{ (pmol/}\mu\text{l or } \mu\text{M)},$$

Where: **Sa** is the sample amount from your standard curve (in pmol),

Sv is sample volume (µl).

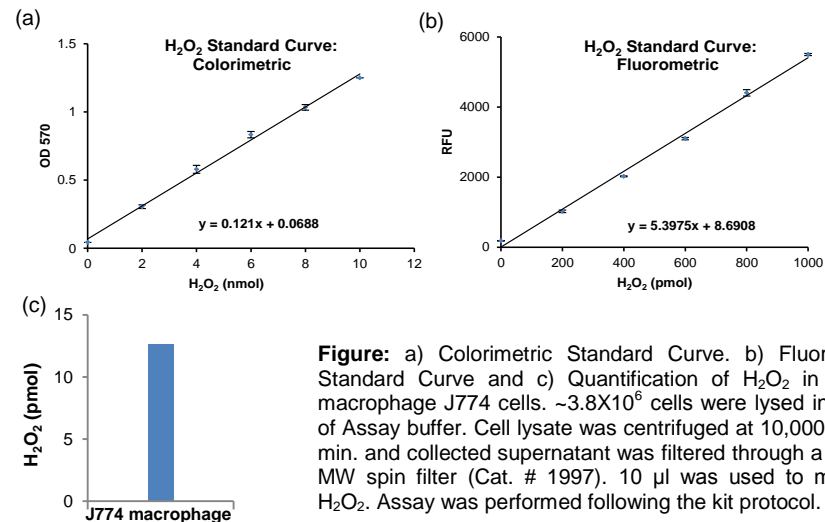


Figure: a) Colorimetric Standard Curve. b) Fluorometric Standard Curve and c) Quantification of H₂O₂ in murine macrophage J774 cells. ~3.8X10⁶ cells were lysed in 380 µl of Assay buffer. Cell lysate was centrifuged at 10,000xg for 2 min. and collected supernatant was filtered through a 10 kDa MW spin filter (Cat. # 1997). 10 µl was used to measure H₂O₂. Assay was performed following the kit protocol.

VI. RELATED PRODUCTS:

Glutathione Reductase Assay Kit
Colorimetric Glutathione Detection Kit (GSH, GSSG and Total)
GST Colorimetric Assay Kit
Acid Phosphatase Assay Kit
Phosphate Fluorescence Assay Kit
NAD/NADH Quantification Kit

Glutathione Peroxidase Assay Kit
ApoGSH Glutathione Detection Kit
GST Fluorometric Assay Kit
Triglyceride Assay Kit
ADP/ATP Ratio Assay Kit
Phosphate Colorimetric Assay Kit
NADP/NADPH Quantitation Kit

FOR RESEARCH USE ONLY! Not to be used on humans.

GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	<ul style="list-style-type: none"> • Use of ice-cold assay buffer • Omission of a step in the protocol • Plate read at incorrect wavelength • Use of a different 96-well plate 	<ul style="list-style-type: none"> • Assay buffer must be at room temperature • Refer and follow the data sheet precisely • Check the wavelength in the data sheet and the filter settings of the instrument • Fluorescence: Black plates ; Luminescence: White plates; Colorimeters: Clear plates
Samples with erratic readings	<ul style="list-style-type: none"> • Use of an incompatible sample type • Samples prepared in a different buffer • Samples were not deproteinized (if indicated in datasheet) • Cell/ tissue samples were not completely homogenized • Samples used after multiple free-thaw cycles • Presence of interfering substance in the sample • Use of old or inappropriately stored samples 	<ul style="list-style-type: none"> • Refer data sheet for details about incompatible samples • Use the assay buffer provided in the kit or refer data sheet for instructions • Use the 10 kDa spin cut-off filter or PCA precipitation as indicated • Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope • Aliquot and freeze samples if needed to use multiple times • Troubleshoot if needed, deproteinize samples • Use fresh samples or store at correct temperatures till use
Lower/ Higher readings in Samples and Standards	<ul style="list-style-type: none"> • Improperly thawed components • Use of expired kit or improperly stored reagents • Allowing the reagents to sit for extended times on ice • Incorrect incubation times or temperatures • Incorrect volumes used 	<ul style="list-style-type: none"> • Thaw all components completely and mix gently before use • Always check the expiry date and store the components appropriately • Always thaw and prepare fresh reaction mix before use • Refer datasheet & verify correct incubation times and temperatures • Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	<ul style="list-style-type: none"> • Use of partially thawed components • Pipetting errors in the standard • Pipetting errors in the reaction mix • Air bubbles formed in well • Standard stock is at an incorrect concentration • Calculation errors • Substituting reagents from older kits/ lots 	<ul style="list-style-type: none"> • Thaw and resuspend all components before preparing the reaction mix • Avoid pipetting small volumes • Prepare a master reaction mix whenever possible • Pipette gently against the wall of the tubes • Always refer the dilutions in the data sheet • Recheck calculations after referring the data sheet • Use fresh components from the same kit
Unanticipated results	<ul style="list-style-type: none"> • Measured at incorrect wavelength • Samples contain interfering substances • Use of incompatible sample type • Sample readings above/below the linear range 	<ul style="list-style-type: none"> • Check the equipment and the filter setting • Troubleshoot if it interferes with the kit • Refer data sheet to check if sample is compatible with the kit or optimization is needed • Concentrate/ Dilute sample so as to be in the linear range
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		